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DEVELOPMENT AND APPLICATION OF AN ENZYMATIC HYDROLYSIS TEST TO ASSESS THE BIODEGRADABILITY OF ORGANIC WASTE MATERIAL

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SUMMARY: A novel and rapid biodegradability test method has been developed based on the enzymatic hydrolysis of cellulose. The test method consists of three phases, in which the first two phases consist of the pH buffer addition, and then autoclaving of the mixture and the final phase is the addition of the enzyme mixture and incubation. An initial investigation was carried out to determine the optimum conditions for the enzymes using standard commercial cellulose as the substrate. The optimised test was then applied to a wide range of organic waste samples including untreated and treated MSW derived mixed BMW, and specific wastes such as waste wood, packaging waste (cardboard), turkey feathers and green waste. The DOC released by enzymatic hydrolysis indicates that this could give an indication of the sample biodegradability. However the DOC released in phases 1 and 2 may also contain some biodegradable components (depending on the extent of biological treatment applied to the waste sample) and these would need to be differentiated from the non-biodegradable DOC and used together with the DOC from phase 3 to give the best possible biodegradability indication.

1. INTRODUCTION

The EU Landfill Directive 31/1999/EC requires that the amount of biodegradable municipal waste (BMW) disposed in landfill be progressively reduced. In the UK the amount of BMW sent to landfill must be reduced to 75%, 50% and 35% of the 1995 baseline by 2010, 2015 and 2020 respectively (Council of the European Union 1999).

Organic waste can be treated to reduce the BMW content in processes such as mechanical-biological treatment (MBT), which is a generic term to describe the process of mechanically sorting and shredding the waste, and then biologically treating the waste by means of composting or anaerobic digestion (Archer, Baddeley *et al.* 2005). Monitoring such processes may be required to assist with maintaining optimal performance and possibly determination of the amount of BMW diverted from landfill following treatment (Environment Agency 2005). This

may include monitoring the input and output waste samples for biodegradability using suitable biodegradability tests.

Such tests may either be conducted over a few days and assess the initial organic matter decomposition rate or be conducted over many weeks until decomposition ceases and the extent of decomposition is measured. The degree in which the rate of biodegradability of the waste is reduced by the process, and the extent of decomposition achieved, can both be used as an indication of the performance and efficiency of the treatment process.

Guidance on the monitoring of MBT processes has been provided for England and Wales (Environment Agency 2005). The Environment Agency specifies two biodegradability test methods, the 100 day anaerobic test (BM100) and the 4 day aerobic test (DR4). The anaerobic test method has been reported to show good reproducibility between results (Godley, Lewin *et al.* 2003), but has the disadvantage of taking a very long time to complete. Short-term aerobic methods like the DR4 test have other disadvantages such as preferentially decomposing the readily biodegradable components of the waste (Godley, Lewin *et al.* 2007) and high microbial growth efficiency such that much of the decomposed organic matter is transformed to microbial biomass rather than mineralized. Therefore most current biodegradability test methods have limitations, and no one test method may be suitable for the whole range of biodegradability testing requirements such as monitoring MBT process performance and assessing organic waste biostability.

A large proportion of BMW consists of biopolymers (proteins, nucleic acids, fats and polysaccahrides) that undergo enzymatic hydrolysis to soluble monomers during the microbial decomposition process before the organic waste is utilized by the microbes as a carbon and energy source. Lignin is an aromatic based polymer that is degraded by oxidative enzymes before utilization by microbes. Lignin is closely associated with cellulose in native plant matter as lignocelluloses and this may comprise 30-50% of organic MSW (Rodriguez, Hiligsmann *et al.* 2005). Agricultural crop waste and forestry residues consist of up to 75-80% cellulose and hemicellulose (Adsul, Bastawde *et al.* 2005). Hemicellulosic/cellulosic material is considered as the most important carbon source for methanogenesis in landfills as it contributes to 90% of the total biogas (CO_2 + CH_4) produced (Rodriguez, Hiligsmann *et al.* 2005). As a general rule, the higher the cellulose/hemicellulose content, the higher the biogas yield of the waste in anaerobic tests (Eleazer, Odle III *et al.* 1997), although the availability of the cellulose can vary as the associated lignin can 'protect' the cellulose from chemical or enzymatic decomposition. Therefore assessment of the waste cellulose and hemicellulose content may provide a non-biological test method of assessing biodegradability.

Direct chemical measurement of the cellulose and hemicellulose content of a waste sample might be considered to give an estimate of the biodegradability of that sample, however this is inappropriate as not all the cellulose is amenable to biodegradation when present as lignocellulose (Chen, Knappe *et al.* 2004). The resistance of lignin to biological and chemical degradation allows it to protect cellulose (Stinson and Ham 1995), and so not all the cellulose picked up in a direct measurement will be biodegradable cellulose. In the lignocellulosic material, lignin may present a physical barrier preventing cellulolytic enzymes from hydrolyzing the cellulosic material (Chen, Knappe *et al.* 2004). Lignin is also considered to be poorly biodegradable under anaerobic conditions (Chen, Knappe *et al.* 2004; Sjöberg, Nilsson *et al.* 2004; Stinson and Ham 1995; Tuomela, Vikman *et al.* 2000).

Following reviews of the current methods (Godley, Lewin *et al.* 2004; Wagland, Tyrrel *et al.* 2007) is has been concluded that there is a need for a rapid and cost-effective test method that would correlate with longer-term tests such as the anaerobic BM100 method. The BM100 test method is not suitable for regular routine testing due to its duration, however a correlating method could make routine testing viable. Cellulose and hemicellulose are hydrolyzed by cellulase and hemicellulase enzymes respectively and so a novel method based on the enzymatic

hydrolysis of cellulytic material could offer a suitable routine test method.

Here the development of the novel enzymatic hydrolysis test (EHT) method is described, and the method is applied to a wide range of organic waste samples.

2. METHODS

2.1 Waste samples

Organic waste samples were collected from a wide range of treatment processes and specific waste streams in the UK as part of the Defra sponsored R&D Waste characterisation project WRT220 (Table 1). The samples ranged from general household waste (BMW), garden waste, wood waste and packaging waste. Where possible the samples were collected pre-, during and post- treatment by either MBT or a mechanical thermal (autoclave) treatment.

The waste samples were sorted to remove glass, metals, plastics and inert materials and only the biodegradable material retained and tested. Materials with large particle sizes were shredded to <10 mm before testing. The dry matter (DM) and loss-on-ignition (LOI) was determined for this sample using standard procedures (EN12879:2000).

2.2 Enzymatic Hydrolysis Test

The enzyme test method consists of three phases of measurement as follows:

- Phase 1- 5 g of LOI is placed in a 250 ml Erlenmeyer flask. 100 ml 0.37 M phosphate pH buffer is then added to the flask and mixed. A 5 ml sample was removed and filtered to remove any solids, and the filtered liquid was then analysed for chemical oxygen demand (COD).
- Phase 2- The sample mixture was then autoclaved at 121°C for 15 min to sterilise the mixture and a further 5 ml sample was removed, and filtered, for COD analysis.
- Phase 3- 20 ml of the prepared enzyme solution was then added to each of the flasks and the flask sealed with a neoprene bung. The flasks were placed in a shaking incubator at 150 rpm. A 5 ml sample was removed for COD analysis, at times specified in later sections.

The amount of moisture in the waste sample and the removal of both the liquid and solids at each stage of sampling, along with the addition of liquid in phase 3, were accounted for in the concentrations of carbon calculated. Soluble COD analysis results were converted to DOC (mg C/l) by assuming a COD/C ratio of 2.67 and then expressed in terms of mg of carbon per kg of the sample (LOI) to give the final values.

2.2.1 Enzyme Preparation

For each sample, 25 mg of crude cellulase powder (Sigma) and 75 mg of hemicellulase powder (Sigma) were dissolved in 20 ml of distilled water, with approximately 175 units' cellulase and 112.5 units' hemicellulase activities in each 20ml of enzyme mixture. This enzyme solution was then filtered through 0.22 μm Millipore membrane filters to sterilise the solution.

The crude cellulase enzymes also possessed some hemicellulase and protease activity, with the hemicellulase enzymes also having some cellulase activity (manufacturer specifications).

2.2.2 Optimisation of the Enzymatic Hydrolysis Test

A commercially available cellulose preparation was chosen for test optimisation (α-cellulose,

Sigma). The pH of the buffer solution was varied at values of 4, 4.5, 5, 5.5 and 6 to determine the optimal pH for the enzymatic hydrolysis. Tests at each pH were carried out in triplicate and the reported results are the mean values.

The effect of temperature was also investigated at each pH value to determine the optimum temperature for enzyme hydrolysis. Temperatures used were 30, 40, 50 and 60°C.

During the Phase 3 enzymic hydrolysis flasks were incubated for at least 60 hours and 5 ml samples taken from the mixture at regular intervals for analysis.

2.2.3 Sample Analysis

The opimal enzymic hydrolysis test conditions of pH 4.75 and temperature 50^{o}C determined using the commercial cellulose (see Section 3.1) was then applied to the collected organic waste samples. In Phase 3 of the test the enzymic hydrolysis incubation period was stopped after 20 h.

3. RESULTS AND DISCUSSION

3.1 Optimisation of the Enzymatic Hydrolysis Test

For the optimisation work, only the DOC released from the enzymic hydrolysis Phase 3 was considered as the amount of DOC released during Phases 1 and 2 for the commercial cellulose was low and amounted to only about 3% of the DOC released by enzyme hydrolysis. Hence Phase 2 results are deducted from Phase 3 values to give only the DOC released by enzymes.

The rate of DOC released from enzyme hydrolysis is initially rapid but then declines until the DOC release stabilises. The purpose of this test is to mimic a biological test and the shapes of the curves is similar to BM100 biogas production curves. The implications being that the EHT may have released a similar amount of DOC that would be decomposed in biological tests suggesting that the test may mimic biological tests that determine the extent of biodegradation. Whether this is the case and whether the initial hydrolysis rate mimics short term biodegradation tests such as the DR4 method (which also measures a rate) is under further evaluation.

Ideally the DOC released in the enzymic test should occur rapidly and be reproducible in order to provide a rapid alternative to long-term tests such as the BM100 method. This may depend on the optimum pH of the enzyme and the impact of temperature on the enzyme activity.

Figures 1-4 indicate that the optimum pH was between 4.5 and 5 at each respective temperature as these pH levels gave the highest initial rate of hydrolysis and the highest overall DOC yield. The time for DOC release to cease was reduced as the temperature was raised, for example at 30°C (Fig. 1) DOC release was still occuring after 75 h but at 60°C (Fig. 4) the DOC release effectively stopped at around 20 h. At 50°C (Fig. 3), the hydrolysis had almost ceased at around 20 h, and the DOC yield was higher at this time than at the same point in the 60°C test. This indicates that significant enzyme denaturation may have limited the amount of DOC released at 60°C. At 40°C (Fig. 2), the hydrolysis rate was lower and DOC release was still occurring after 70 h, and although the highest DOC yield was at 40°C and pH 5 (90285 mg C/kg LOI), it would take a long time to reach the end point, in comparison to 50 and 60°C. At 60°C the highest DOC yield was lower (pH 4.5- 47000 mg C/kg LOI) than the highest DOC yield at 50°C (pH 4.5- 67000 mg C/kg LOI). From the graph of 50°C, it is evident that about 85% of the total DOC released after 100 h is released after only 20 h. Therefore, although the amount of DOC released at 50°C is lower than at 40°C, the timescale is much more rapid. The optimal test conditions chosen were therefore pH 4.75, temperature 50°C and a 20 hour incubation time as a compromise between enzyme denaturation and a rapid hydrolysis rate so that the test time-scale was reduced to less than a day and much lower than most biological tests.

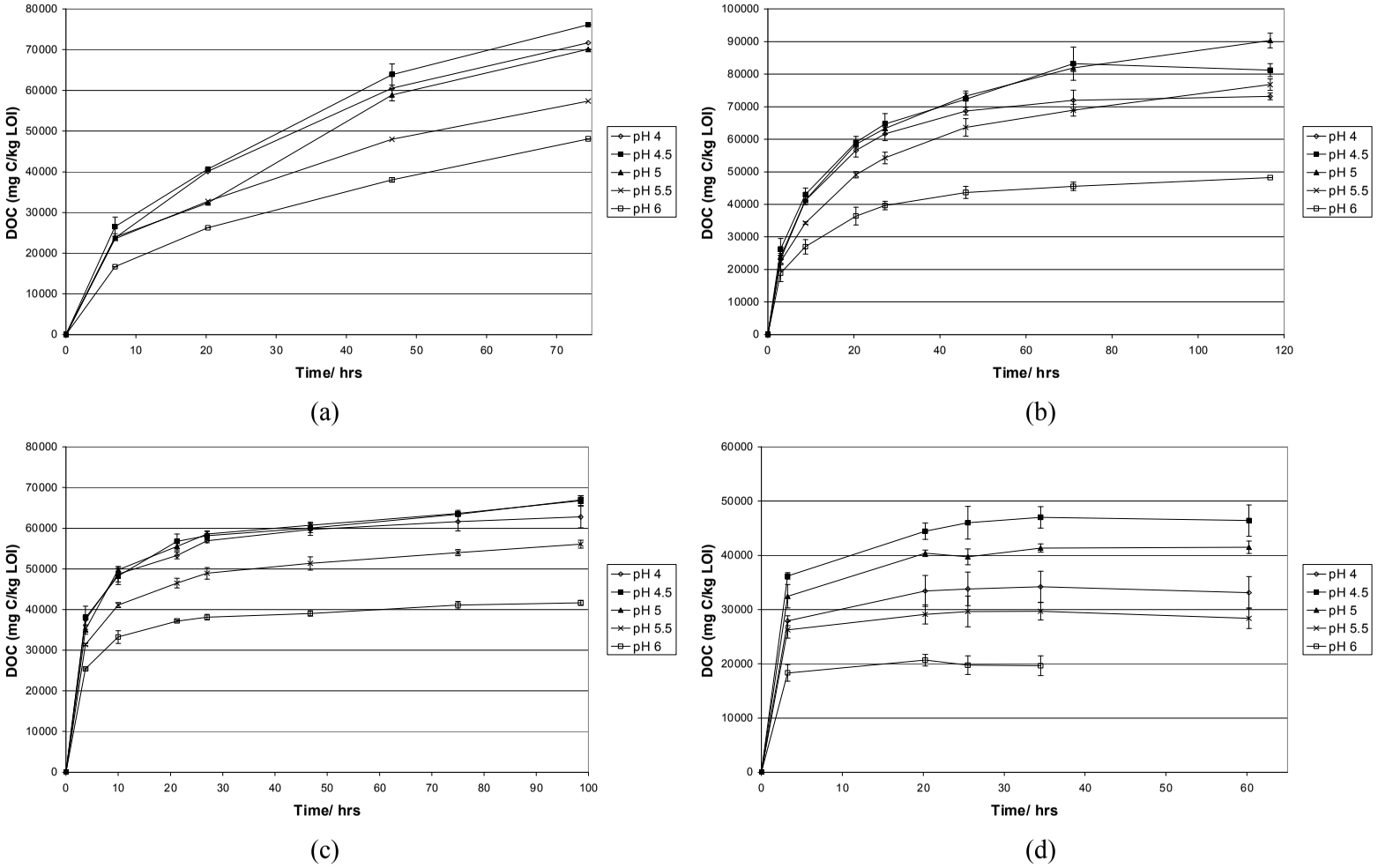

Figure 1. DOC (mg C/kg LOI) increase over time at incubation temperature 30°C (a), 40°C (b), 50°C (c) and 60°C (d). Error bars shown as standard deviation (stdev).

3.2 Waste Samples

The waste samples selected for this study included samples before and after MBT treatment (composting and anaerobic digestion), composting and autoclave treatment to demonstrate the effects of treatment to the DOC obtained in the test at each stage. The samples included packaging waste (cardboard), waste wood, garden waste, household waste (mixture of kitchen and garden waste), turkey feathers and MSW derived mixed BMW.

The cumulative DOC released after each phase of the test varied greatly between the samples (Table 1). The results are expressed as mean values of the three replicates analysed. Much more DOC was released during Phases 1 and 2, before the enzyme hydrolysis Phase 3, in many of the waste samples compared with commercial cellulose. Phase 1 DOC may represent the low molecular weight readily soluble materials present in the waste, whilst the DOC released in Phase 2 may represent soluble DOC following mild acid hydrolysis of some of the polymeric components during autoclave. Phase 2 DOC may also include soluble materials desorbed from the waste during the autoclaving. Finally the DOC released in Phase 3 is due to the enzymatic hydrolysis of the material, and so may indicate the amount of additional biodegradable cellulose, hemicellulose and possibly proteinaceous material present. The DOC released at each phase is shown graphically in Figure 5.

Table 1. Results of DM, LOI and the Enzymatic Hydrolysis Test (DOC released at the end of each phase).

	DM	LOI	DOC (mg C/kg LOI)		
Sample	**% wet wt**	**% DM**	**Phase 1**	**Phase 2**	**Phase 3**
Commercial cellulose	96.5	98.4	779	1550	53500
Construction wood waste	77.5	91.4	1500	13000	16000
Autoclaved construction wood waste	63.9	90.7	5380	15900	17800
Packaging waste	42.6	93.6	960	3540	26300
Autoclaved packaging waste	40.4	93.1	3080	6350	22900
Greenwaste (untreated)	40.4	73.2	10000	26400	32100
Partially composted greenwaste	44.2	62.1	11500	25100	33200
Kitchen and greenwaste (untreated)	35.1	65.3	11300	26000	34800
Partially composted kitchen and greenwaste	38.3	68.0	7250	16900	17900
Composted kitchen and greenwaste	51.7	60.9	3870	25400	26800
Organic fibre from autoclaved MSW	50.5	76.9	12000	29800	44400
AD treated fibre from autoclaved MSW	29.2	72.5	1640	7160	7900
Turkey feathers	33.7	99.3	8290	12000	16700
Autoclaved turkey feathers	38.3	96.2	7950	28100	32000
Stabilised greenwaste compost <10 mm	71.9	29.2	3450	47100	55500
Stabilised greenwaste compost <25 mm	66.6	28.9	3970	57700	58200
MSW input windrow MBT	94.4	73.4	13500	33800	61100
Fully Composted MSW	66.3	29.6	1310	28300	30600
MSW input to AD	96.5	58.7	17400	80800	141000
Output MSW AD	31.5	56.2	685	7570	8600
Fresh MSW	94.4	39.3	14300	80300	104000
Composted MSW <15 mm	75.3	23.4	9710	41600	47200

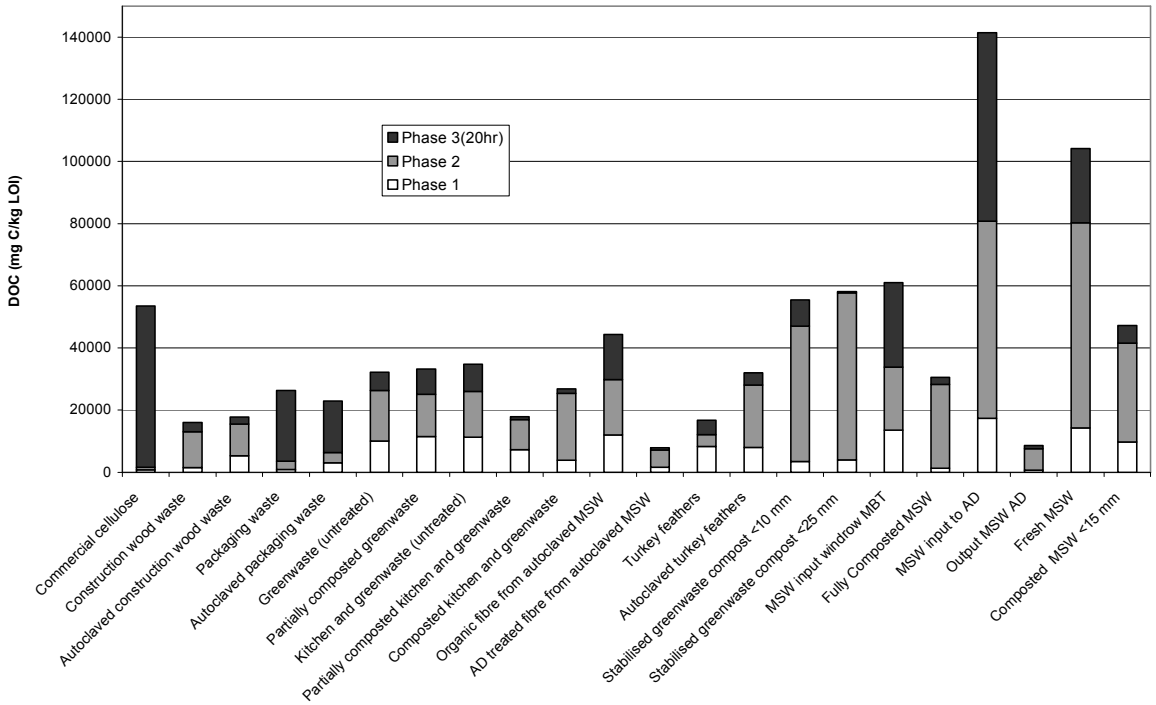

Figure 5. DOC released at each phase of the Enzymatic Hydrolysis Test.

The non-enzymatic DOC (Phases 1 and 2) for wastes that have undergone extended biological treatment (e.g. the fully composted greenwaste and composted MSW derived BMW samples), are likely to consist of significant amounts of humic substances resulting from the decomposition of lignin (Stevenson 1994). These substances are not usually considered readily biodegradable, and so in these cases, the DOC due to enzymatic hydrolysis (Phase 3 only) may be indicative of the sample biodegradability. Unlike the control cellulose, many of the non-biologically treated (raw or autoclaved) waste samples also showed significant amounts of DOC released during Phases 1 and 2. As these wastes have not been biologically treated it seems reasonable to assume that much of the DOC released during Phases 1 and 2 will be inherently biodegradable. Therefore a key question regarding this data is whether the test result should include either the entire DOC released, or the DOC released through enzymatic hydrolysis alone (Phase 3 only).

As part of Defra project number WRT220, BM100, DR4 and biochemical data is available (Godley, Frederickson *et al.* 2007; Godley, Lewin *et al.* 2007) for the majority of these samples. A full interpretation of these results is in preparation, but preliminary analysis of the data indicates that the EHT test method shows good correlation wth the BM100 test results for many of the samples.

For example, the packaging waste and autoclaved packaging waste both have a high proportion of cellulose (42.6 – 46.2% of dry matter) and gave high biogas production values in the BM100 test (527 – 630 l/kg LOI). In the EHT the major fraction of DOC was released during Phase 3 in both samples, which is expected given the high cellulose content of these samples.

The organic fibre from autoclaved MSW and the AD treated fibre from MSW both contain a relatively high fraction of solubles (35% and 27% respectively). Therefore the high DOC contribution observed for Phases 1 and 2 (Fig. 6) in the EHT is expected, and the actual values (Fig. 5) are consistent with the soluble fraction differences. The AD treated fibre from MSW has

under half the cellulose fraction and over double the lignin faction of the organic fibre from autclaved MSW. Therefore the lower DOC from enzymatic hydrolysis (Phase 3) observed (Fig. 6) for the AD treated sample might then be expected.

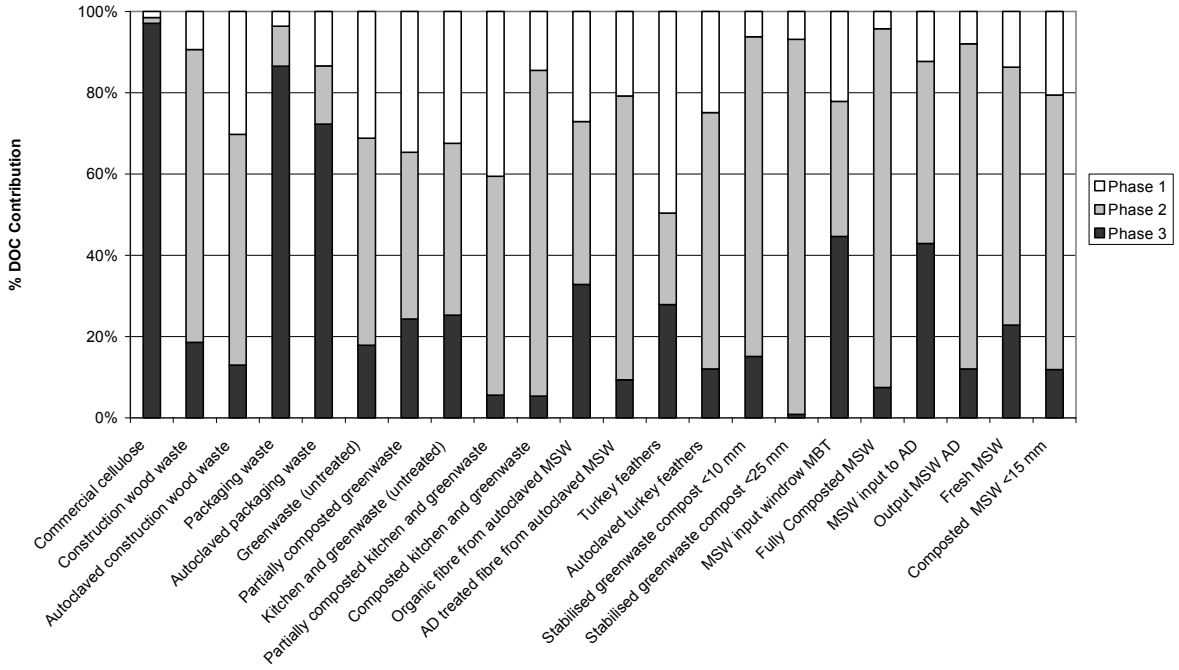

Figure 6. Percentage contribution of each phase to the total DOC released.

In general the results indicate that significant amounts of DOC are released by the EHT test during Phases 1 and 2 before the enzyme is introduced into the tests. For untreated waste samples much of this material may be biodegradable and for fully biologically treated wastes this material may be recalcitrant. The DOC released during Phases 1 and 2 for partially biologically treated materials may be composed of mixtures of biodegradable and recalcitrant materials. Therefore in order to successfully assess the biodegradability of a sample the DOC due to enzymatic hydrolysis alone is not sufficient, although it may give a good indication. The biodegradable DOC from Phases 1 and 2 may need to be differentiated from the non-biodegradable DOC and used together with the DOC from Phase 3 to give the best possible biodegradability indication.

4. CONCLUSIONS

From this work it is concluded that the enzymatic hydrolysis test shows good potential as a novel and rapid (sub 24 hour) biodegradability test method. Further work is progressing to fully evaluate this test method, including consideration of the impact of pre-biological treatment on the biodegradability of DOC released during Phases 1 and 2 of the test and the application of the test to a wider range of samples and comparison with the results obtained from the BM100 and DR4 test methods.

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